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INTRODUCTION

At the time of diagnosis, 30% of all breast tumors grow independently of hormone. These cancers are unlikely to respond to currently available hormonal therapies. Of the 70% of breast tumors that do depend on hormone for growth, only about half of these patients will respond to hormonal therapies, such as aromatase inhibitors and the selective estrogen receptor modulator (SERM) tamoxifen. However, drug resistance often develops after long term treatment as the tumor progresses to a hormone-independent state. There is a great need for novel therapies for breast cancer.

Estrogen-related receptor alpha (ERRα) is a protein that is structurally and functionally similar to estrogen receptor alpha (ERα), the primary focus of hormonal therapy for breast cancers. Like ERα, ERRα can bind to estrogen-response elements (EREs) present in certain gene promoters and, by interacting with transcriptional co-activators, activate transcription. But unlike ERα, ERRα binds neither estrogen nor tamoxifen; in fact, all data indicate that ERRα can act independently of hormones. Recent statistical data from a retrospective clinical study indicate that over-expression of ERRα correlates with breast cancer recurrence and adverse clinical outcome (Suzuki *et al*., 2004). In addition, using quantitative RT-PCR analysis in clinical primary breast cancers, expression of ERRα is correlated with ERα-negative and HER2-positive tumors status (Ariazi *et al*., 2002). These observations strongly suggest that ERRα plays an important role in the progression of a significant subset of breast cancers.

We hypothesize that ERRα may be a new candidate for diagnostic and therapeutic strategies. We believe disruption of certain ERRα-co-activator interactions by a small molecule will decrease transcriptional activation of ERRα target genes, some of which are involved in proliferation, anti-apoptosis, and progression of breast cancers. The goal of this project is to find a new treatment for human breast cancer, particularly for the subset of aggressive breast cancers that are hormone-independent and/or resistant to currently available therapies.

RESEARCH ACCOMPLISHMENTS

The three tasks of our approved Statement of Work were the following:
Task 1 - <u>Determine ERRα-associated proteins by using gentle immunoaffinity chromatography and identify the proteins using MALDI-TOF mass spectrometry;</u>
Task 2 - <u>Study the binding properties of ERRα and a co-activator</u>; and
Task 3 - <u>Develop a high-throughput screen (HTS) for molecules that interfere with the binding of ERRα and a co-activator</u>.

Discoveries made during Year 2 of the grant period (as part of a collaboration between our laboratory and the Mertz laboratory) identified a constitutively-activated form of ERRα and a clearly relevant co-activator (glucocorticoid receptor interacting protein 1, GRIP1). As a result we chose to bypass Task 1 and to focus heavily on Tasks 2 and 3. We have since made major progress in developing a cell-based, *in vivo* high-throughput screen, carrying out our first screening of a chemical library, generating even more efficient large-scale screens, carrying out additional screens of chemical libraries, and developing counter-screens. Below we describe the progress made during the three years of this grant support, focusing on the third and last year of the granting period, as well as future plans to carry out counter-screens, further cell-based screens, target-based high-throughput screens, and biological assays to characterize the resulting lead compounds.

We anticipate that results from these studies will contribute to the understanding of the transcriptional control of breast cancer and to a new therapeutic approach for treating some breast cancers. This grant has allowed us to progress toward identifying and characterizing promising lead compounds. As we further progress, we hope to initiate collaboration with a suitable pharmaceutical

company to facilitate the transfer of the project out of the university and into an environment more appropriate for further development of the drug toward pre-clinical and clinical trials.

Task 1- Determine ERRα-associated proteins.

Because of our discoveries and progress during Year 2 of the grant (see Figure 3 from March 2007 annual report), we chose not to finish the work described in the original Task 1, which was to develop monoclonal antibodies to ERRα, and then use these antibodies in immunoaffinity chromatography to isolate and identify relevant co-activators of ERRα for use in Tasks 2 and 3. Since we had identified the p160 family member, GRIP1, as a specific co-activator of ERRα-dependent, ERE-regulated transcription in MCF-7 mammary carcinoma cells, we felt justified in moving directly to high-throughput screen development and use. However, we found many uses for the monoclonal antibodies we had already prepared in Task 1. We thought it was important to continue to develop and extensively characterize monoclonal antibodies against ERRα during this entire granting period. We have also found a need for monoclonal antibodies against GRIP1 during this past year, so we have made progress in generating hybridoma cell lines from cells isolated from mice that had been immunized with purified GRIP1.

➢ Generation and characterization of murine monoclonal antibodies against ERRα.

Cross-reactivity of immunological reagents is a significant problem when studying the biology of members within a protein family. Thus, it is very important for us to have well-characterized monoclonal antibodies to ERRα. The details of the generation of these monoclonal antibodies have been described in detail in our past two progress reports. Briefly, cDNA of ERRα1 was inserted into the overexpression plasmid pET28b(+) (Novagen). The human ERRα1 gene with a C-terminal hexahistidine-tag was encoded and expressed from a T7 promoter in *Escherichia coli* and purified by a nickel-nitrilotriacetic acid (Ni-NTA) affinity column. Using standard hybridoma techniques, a panel of murine mAbs was produced to the hexahistidine-tagged ERRα. Nine of those mAbs were tested for specificity in a Western blot.

We were then able to characterize these antibodies for use in a Western blot, and we were able to roughly determine the epitope of eight of the nine mAbs. These mAbs were subsequently used in many assays by other members in the laboratory. The mAbs were used in: immunoprecipitation experiments for isolating over-produced ERRα in cell extracts; supershift assays, which are similar to non-denaturing gel shift assays but instead tests an antibody's ability to bind radiolabeled DNA-bound protein; immunofluorescence assays to determine ERRα's sub-cellular distribution in a variety of mammary, cervical, and liver cell lines fixed to glass slides; and chromatin immunoprecipitation (ChIP) assays to immunoprecipitate DNA-bound, overproduced or endogenous ERRα. In our annual report for March 2006, we included a table titled, "Summary of anti-ERRα monoclonal antibody characterization." We have reproduced this table for this final report, italicizing new or updated information (see Table 1 below).

These mAbs are now being used in ChIP – chip assays to determine novel biomarkers under the transcriptional control of ERRα, but not ERα (Esch, unpublished). Also, one specific monoclonal antibody, 1ERR87, was used to immunoprecipitate ERRα, which assisted in determining novel phosphorylation and sumoylation sites at the N-terminus of ERRα in MCF-7 mammary carcinoma cells (Vu *et al.*, 2007).

We are excited to report that the nine ERRα monoclonal antibodies are commercially available; they are licensed through the Wisconsin Alumni Research Foundation (WARF) to Santa Cruz Biotechnologies and to Sigma-Aldrich Israel.

Table 1. Characterization of ERRα monoclonal antibodies.

mAb	1ERR21	1ERR87	1ERR90	2ERR1	2ERR2	2ERR6	2ERR7	2ERR10	2ERR37
Purified?	Yes	Yes	No	Yes	No	Yes	No	Yes	Yes
Isotype	IgG_1	IgG_1	IgG_1	IgG_{2a}	IgG_{2a}	IgG_{2b}	IgG_{2b}	IgG_1	IgG_1
WB	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Epitope	199-376	1-76	1-76	nd	*1-76*	*144-423*	199-376	1-76	*77-198*
IP	Yes	Yes	nd	nd	nd	No	nd	Yes	nd
Supershift	No	Yes	Yes	Yes	nd	No	No	Yes	No
IF	*No*	*Yes*	*Yes*	*Yes*	*Yes*	*No*	*No*	*Yes*	*Yes*
ChIP	*Yes*	*Yes*	*No*	*Yes*	*nd*	*No*	*No*	*Yes*	*Yes*

ERRα: A 423-amino acid nuclear receptor protein related to estrogen receptor.
Purified: purified from ascites fluid using methods previously described in Thompson and Burgess (2001).
Isotype: Type of immunoglobulin heavy chain; antibody subtype.
WB: Western blot; suitable for use in immunoblotting to detect protein expression.
Epitope: Location of mAb binding site on full-length ERRα (amino acids 1-423); region from amino acid # to amino acid #.
IP: Immunoprecipitation; suitable for use in IP to isolate ERRα and any associated proteins from cell extracts.
Supershift: Able to detect ERRα in its DNA-bound form in a non-denaturing gel shift assay.
IF: Immunofluorescence; suitable for use in IF to determine sub-cellular distribution of ERRα in cells.
ChIP: chromatin immunoprecipitation; able to immunoprecipitate ERRα in its chromatin-bound form from cell extracts.
nd: not determined.

> Generation of murine monoclonal antibodies against GRIP1.

As mentioned in our last annual report (March 2007), we felt there was a great need for a good commercially available monoclonal antibody against GRIP1. Dr. Michael Stallcup of the University of Southern California generously provided us with glutathione-S-transferase (GST)-tagged GRIP1, which we were able to partially purify from *E. coli* Rosetta2(DE3)pLysS extract.

This past year, we used this GRIP1 protein as an immunogen to produce murine monoclonal antibodies against GRIP1. We screened the resulting hybridoma cells for clones that produced monoclonal antibody against GRIP1. We were able to further clone some hybridomas that seemed promising, but unfortunately, we were unable to isolate a true hybridoma cell that would reliably produce GRIP1 mAbs. We are now working hard to purify a new batch of GST-tagged GRIP1, but this time to homogeneity by additionally using size-exclusion and ion-exchange chromatography. We are hopeful that we may isolate one or more true monoclonal hybridoma cell lines that will provide us with antibodies against GRIP1. The resulting monoclonal antibodies will be made widely available.

Task 2 - Study the binding properties of ERRα and co-activator.

As described in the last annual report (March 2007), the Mertz laboratory determined that a truncated form of ERRα acts as a constitutive activator of transcription and found that GRIP1 can specifically co-activate transcriptional activity of truncated ERRα on an ERE-containing promoter reporter gene. From these observations, we had a way to discover specific inhibitors of ERRα-co-activator complexes involved in the transcriptional regulation of genes contributing to breast cancer. Therefore, we focused on developing and optimizing a cell-based high-throughput screen to find such small molecule inhibitors. This cell-based screen relies on the inhibition of ERRα-GRIP1-dependent luciferase expression; consequently, there is a significant risk of a variety of classes of false-positive chemical hits.

We needed to create an assay that would determine if the hits identified in the cell-based, high-throughput screen inhibited the ERRα-GRIP1 interaction specifically. Therefore, we are developing a

protein fragment complementation assay (PCA), which measures specific protein-protein interactions in a cellular environment (adapted from the method of Remy and Michnick, 2006). The *Gaussia princeps* luciferase is the smallest and brightest known luciferase; it is about 1,000-fold brighter than firefly or *Renilla* luciferases. For the PCA, *G. princeps* is split into two fragments, called hGLuc(1) (amino acids 1-93) and hGLuc(2) (amino acids 94-169). We are presently creating cDNA constructs of GRIP1 and truncated ERRα fused to the hGLuc sequence fragments at either the 5' or 3' end. The luciferase fragments will be tethered to ERRα and GRIP1 via a flexible linker, $(Gly_4Ser)_2$. To optimize *G. princeps* luciferase activity, all four combinations of fusion proteins will be tested in transient transfection assays using MCF-7 cells. Only when ERRα and GRIP1 interact in the cell will the luciferase protein fragments come in close enough proximity to allow enzyme activity that can be measured *in vivo* by addition of its cell membrane permeable substrate, coelenterate luciferin (coelenterazine). *G. princeps* luciferase activity will decrease if the compound interferes with the ERRα-GRIP1 interaction.

We believe this will be a complementary assay to the cell-based high-throughput screens described below in Task 3. Any compound hits resulting from our high-throughput screens and counter-screens will be tested in this PCA; we will pursue any hits in this assay as specific inhibitors of our targeted protein-protein interaction.

Task 3 - Develop a high-throughput screen (HTS) for molecules that interfere with the binding of ERRα and a co-activator.

➢ First generation, primary cell-based HTS.

We used the novel cell-based assay (see Fig. 3 from March 2007 annual report) to scale-up and develop a high-throughput screen, performed in 96-well plates in MCF-7/WS8 cells treated with 10^{-8} M ICI 182,780 (a compound that inhibits ERα; Tocris, Ellisville, MO). Eighteen hours after plating, cells were transiently transfected with three separate DNA vectors containing truncated ERRα, GRIP1, and a firefly luciferase reporter gene downstream of an ERRα-binding sequence containing promoter (five copies in tandem of the vitellogenin ERE, 5'- cttAGGTCAcagTGACCTaag-3'; ERE half-sites are capitalized). Twenty-four hours post-transfection, one microliter of 1 millimolar chemical compound was added to each well; the final compound concentration was therefore 10 micromolar. We used a 4,160 "known bioactives" (KBA) compound library in our pilot screen. At 24 hours post-compound addition, cells were harvested using a mixture of 1:2 ratio of Bright-Glo luciferase reagent to 1.5x Passive Lysis Buffer (Promega, Madison, WI). A well in which a 50% or more decrease in luminescence occurred compared to control was considered a "hit". Of the 4,160 compounds in the KBA library, 548 hits decreased luminescence by >50%, which was ~10%. This was quite high for a screen. Based on other screens being performed on the KBA library, we were able to determine that 348 were shown to be promiscuous inhibitors; therefore, 200 compounds were unique hits to this pilot screen.

➢ Development of a second generation, primary cell-based HTS.

We have improved our first generation, cell-based HTS by expanding our screening to 384-well plates and transfecting cells in bulk. Consequently, we have decreased by 75% the amount of cells and chemicals needed per well, greatly reduced handling time of the plates and changing of pipette tips, and making the cells added to each well more uniform. We have also found ways to significantly reduce the "edge effect", a phenomenon in which sample wells on the edges of 384-well plates experience changes in volume due to evaporation during a 48-hour HTS, which can greatly influence our assay and data analysis. These improvements have greatly decreased well-to-well volume variation

and increased our confidence that final compound concentration (10 micromolar) is present in all wells. Lastly, transfecting cells in bulk allowed us to shorten our total assay time by one day.

➢ Improvements to the second-generation, primary cell-based HTS.

To determine the optimal window of luciferase activity in our second generation screen, we have monitored luciferase activity as a function of hours after transfecting cells in bulk ("hours post-plating/transfection). **Fig. 1** shows a representative time-course experiment. At 48 hours post-plating/transfection, we are able to see a 20- to 50-fold increase in luciferase activity in the presence of truncated ERRα and GRIP1, compared to a control with GRIP1 but no truncated-ERRα.

In our first- and second-generation cell-based HTS, we added compound 24 hours post-transfection of cells and read luciferase activity (luminescence) after 24 hours, with good results. Based on these recent time-course experiments, however, we may be able to amend our second generation screen by adding compound after only 16-20 hours post-bulk transfection of cells and reading luciferase activity after 32-40 hours. We will test to see if this adjustment will improve our ability to detect changes in reporter activity and therefore provide a more sensitive, more rapid assay for detecting compound inhibitors.

Alongside the time course assay, we used known compounds in the second-generation HTS to test the sensitivity and reliability of the assay (**Fig. 2**). We tested various concentrations of inhibitors of replication (actinomycin D), transcription (alpha-amanitin), translation (cycloheximide), and of proteosome activity (MG-132). We also tested a non-specific compound (resveratrol), and the compound XCT-790, which potentiates the degradation of ERRα. Increasing concentrations of all the compounds, except resveratrol, increasingly reduced GRIP1-ERRα-dependent luciferase activity. This experiment showed that our assay could measure reductions in luciferase activity; in addition, this experiment also showed us examples of "false-positive" compound hits.

➢ Implementation of the second generation, primary cell-based HTS.

We moved forward and used this improved second-generation HTS to carry out an initial screen of 9,400 compounds from the DIVERSet subset of the Chembridge library at the Keck-UW Comprehensive Cancer Center Small Molecule Screening Facility (SMSF, *http://chembridge.com/chembridge/compound.html#p*). This compound library is a collection of diverse, pre-designed drug-like small molecules. We have identified 64 hits that inhibit luciferase enzyme activity by more than 75%, and 34 of them that are not generally toxic to cells. We are presently screening the remaining 6,600 compounds in this DIVERSet subset of the Chembridge library, after which we will be able add any compound hits to our current collection of 34 hits. We will then be able to compare the structures of our compound hits.

We would like to use our cell-based HTS to screen larger compound libraries. The remaining libraries offered by the SMSF contain a total of 41,824 compounds. Each of these compounds falls under at least one of the following categories: (1) fits the Lipinski guidelines for "drug-likeness" (Lipinski *et al.,* 2001); (2) has known anti-cancer activities; (3) is a suspected endocrine disrupter; (4) contains novel structural types and has shown unusual patterns of cell line sensitivity and resistance; (5) is a natural product; or (6) is a compound of known safety and bioavailability in humans (adapted from SMSF website, http://hts.wisc.edu/Libraries.htm). Therefore, we are excited about continuing to use the second-generation, cell-based HTS described above on the remaining compound libraries. Hopefully these HTS will generate a sizable collection of primary compound hits.

➤ Cell-based high-throughput counter screen to discard primary compound hits that affect general transcription or translation.

Figure 2 showed us examples of known false-positives in our primary HTS. Because one would expect more examples of false-positive hits, such as those that damage cells or generally affect transcription or translation, we have developed a cell-based, high-throughput counter screen to identify and discard those compounds. We use the same methods as those that had been used for the second-generation, primary, cell-based HTS; however, we omit the ERRα-binding luciferase reporter vector. Instead, we substitute a reporter vector containing an SV40-promoter upstream of firefly luciferase (specifically, the pGL3-promoter vector from Promega). We have confirmed that the presence or absence of GRIP1 and truncated ERRα has no affect on the expression of this firefly luciferase reporter; therefore, equivalent microgram amounts of the SV40-driven luciferase reporter vector are transfected in this counter screen as compared to total DNA transfected in the primary HTS. Luciferase activity should decrease if the compound hit tested interferes with general transcription, RNA processing, translation, or luciferase enzyme activity; consequently, we will discard these non-specific, false-positive compounds. We have the necessary reagents and are presently performing this assay on the 34 compound "hits" from our primary HTS mentioned above.

➤ Biological assays.

The MCF-7/WS8 cells were selected for the HTS because they are easy to culture in standard attachment-dependent cell culture, they are amenable to large-scale transfection procedures, and they show a robust response to activation of gene-specific transcription by co-transfection with ERRα and GRIP1. However, we will want to examine the biological effects of the lead hit compounds on a variety of other breast cancer cell lines in culture.

We are starting to develop these cancer cell line assays; we will monitor the effects of the compounds on various biological functions such as cell proliferation, migration, anchorage-independence, ERRα and GRIP1 protein levels, and ERRα-dependent transcriptional profiling. Our tools for these assays will be not only MCF-7/WS8 cells, but also two other standard breast cancer cell lines, BT-474 and MDA-MB-231. BT-474 cells are ERα-positive/HER2-overexpressing and are considered to be invasive; MDA-MB-231 cells are ERα-negative and are also considered to be invasive (Tong *et al*., 2002). To monitor the effects of the compounds on protein levels, we will also use our panel of monoclonal antibodies developed during this granting period in immunofluorescence assays and by immunoblot analyses to assess ERRα and GRIP1 protein levels in these cell lines; other cell lines will be considered as experiments progress.

KEY RESEARCH ACCOMPLISHMENTS – Entire Granting Period

➢ Discovered that the p160-family member glucocorticoid receptor interacting protein 1 (GRIP1/SRC-2/TIF2/NcoA-2) is a cell type- and promoter-specific co-activator of a constitutively-activated mutant ERRα.

➢ Developed first- and improved second-generation, primary cell-based, high-throughput screens for small molecule inhibitors of ERRα-GRIP1-dependent luciferase expression.

➢ Developed a cell-based counter screen to discard false-positive hits that affect general transcription or translation.

➢ Made technical improvements to increase ease and reproducibility of cell-based HTS.

➢ Generated and extensively characterized nine murine monoclonal antibodies against ERRα. These antibodies are now commercially available.

➢ Continuing in our efforts to generate monoclonal antibodies against GRIP1.

➢ Developing biological assays to determine the effects of our lead compounds on cellular functions, and to further validate their potential as biologically relevant inhibitors of ERRα activity.

REPORTABLE OUTCOMES – Entire Granting Period

➢ **Poster sessions/Abstracts**

• *ERRα – co-activator GRIP1 protein-protein interaction: a target for novel breast cancer therapeutics*? Era of Hope Meeting; Baltimore, MD; June 25-28, 2008.

• *ERRα – co-activator GRIP1 protein-protein interaction: a target for novel breast cancer therapeutics*? Seventh Annual Molecular and Cellular Pharmacology (MCP) – Sponsored Signal Transduction Research Training Symposium; Madison, WI; April 8, 2008.

• *Generation and characterization of murine monoclonal antibodies for use in studying the biology of the estrogen-related receptor alpha (ERRα)*. The Keystone Symposium, Nuclear Receptors: Orphan Brothers; Banff, Alberta, Canada; March 18-23, 2006.

• *Generation and characterization of a set of immunological reagents for use in studying the biology of the estrogen-related receptor alpha (ERRα)*. Great Lakes Nuclear Receptor Conference; Madison, WI; October 15, 2005.

➢ **Presentations**

• Cancer Biology Student/Postdoc Seminar Series; University of Wisconsin – Madison
  - *Generation and characterization of a set of monoclonal antibodies for use in studying the biology of the estrogen-related receptor alpha (ERRα)*. December 20, 2005.
  - *Studying Estrogen-Related Receptor α (ERRα)—Co-activator Interactions in Breast Cancer Cells*. February 1, 2005.

• Molecular and Cellular Pharmacology Student Seminar Series; University of Wisconsin – Madison
  - *ERRα – co-activator GRIP1 protein-protein interaction: a target for novel breast cancer therapeutics*? February 18, 2008.
  - *Estrogen-related Receptor alpha—Co-regulator Interactions as Possible Targets for Novel Breast Cancer Therapies*. January 29, 2007.
  - *Generation and characterization of a set of monoclonal antibodies for use in studying the biology of the estrogen-related receptor alpha (ERRα)*. December 5, 2005.
  - *Studying Estrogen-Related Receptor α (ERRα)—Co-activator Interactions in Breast Cancer Cells*. April 11, 2005.

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➢ **Funding applied for or received based on the work supported by this grant**
- WARF Strategic Technology Enhancement Program Lead Discovery Initiative, 2006-2008.
- Applied for the Susan G. Komen For the Cure Investigator-Initiated Research Grant, Dec 2007 (pending).
- Applied for the National Institutes of Health RO1 Grant, Feb 2008 (pending).
- Graduate School Collaborative, University of Wisconsin – Madison, Vilas Travel Award ($600 award for travel to the Keystone Symposia in 2006).

➢ **Personnel receiving pay from the research effort**
- Jennifer A. Lamberski
- Nancy E. Thompson
- Janet E. Mertz
- Richard R. Burgess

CONCLUSIONS

We have made considerable progress in completing Tasks 1-3 during this granting period. We have been able to generate, characterize, and commercialize nine ERRα monoclonal antibodies. Importantly, others in the laboratory are using these antibodies to answer questions involving the global transcriptional regulation of ERRα, and the post-translational modifications of ERRα, in mammary carcinoma cells. We have established the glucocorticoid receptor interacting protein 1 (GRIP1) as an important co-activator for ERRα on ERE-containing promoter elements in human mammary carcinoma cell lines. We then have purified a GST-tagged version of GRIP1 protein and used it to immunize mice; we are now generating hybridoma cells and screening for monoclonal antibodies to GRIP1. We have used GRIP1 and a truncated, constitutively-activated ERRα (amino acids 77-423) to develop a first- and improved second-generation primary cell-based high-throughput luciferase reporter assay. We are using a SV40 promoter-driven firefly luciferase reporter in a cell-based counter assay to help discard false-positive compound hits. Lastly, we are adapting the protein fragment complementation assay (PCA) using two halves of the *Gaussia princeps* luciferase enzyme, which will allow us to measure GRIP1-truncated ERRα protein-protein interaction *in vivo*.

Because of this funding opportunity, we have developed and implemented assays that will allow us to discover lead compounds that interfere with the functional interaction between ERRα and the co-activator GRIP1. We believe this protein-protein interaction may play an important role in the progression of a subset of presently difficult-to-treat breast cancers, and we are excited to have developed assays that may lead to a potential therapeutic for these cancers.

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SUPPORTING DATA

Figure 1. Time course assay of the change in firefly luciferase activity over time. MCF-7/WS8 cells were transfected in bulk using TransIT-LT1 reagent (Mirus, Madison, WI) and with the following DNA vectors: p(5x)ERE-ffLuc; pcDNA3.1 (the backbone vector, "minus ERR-alpha") or truncated ERRα "plus ERR-alpha"; and GRIP1. Transfected cells were harvested every 8 hours for 48 hours after plating. Media was removed from the wells. Bright-Glo luciferase substrate (Promega, Madison, WI) was added to each well and allowed to incubate for two minutes. Luminescence was read at 700 nm for 1 second per well. Assays are done in quadruplicate. Error bars indicate standard deviation from the mean.
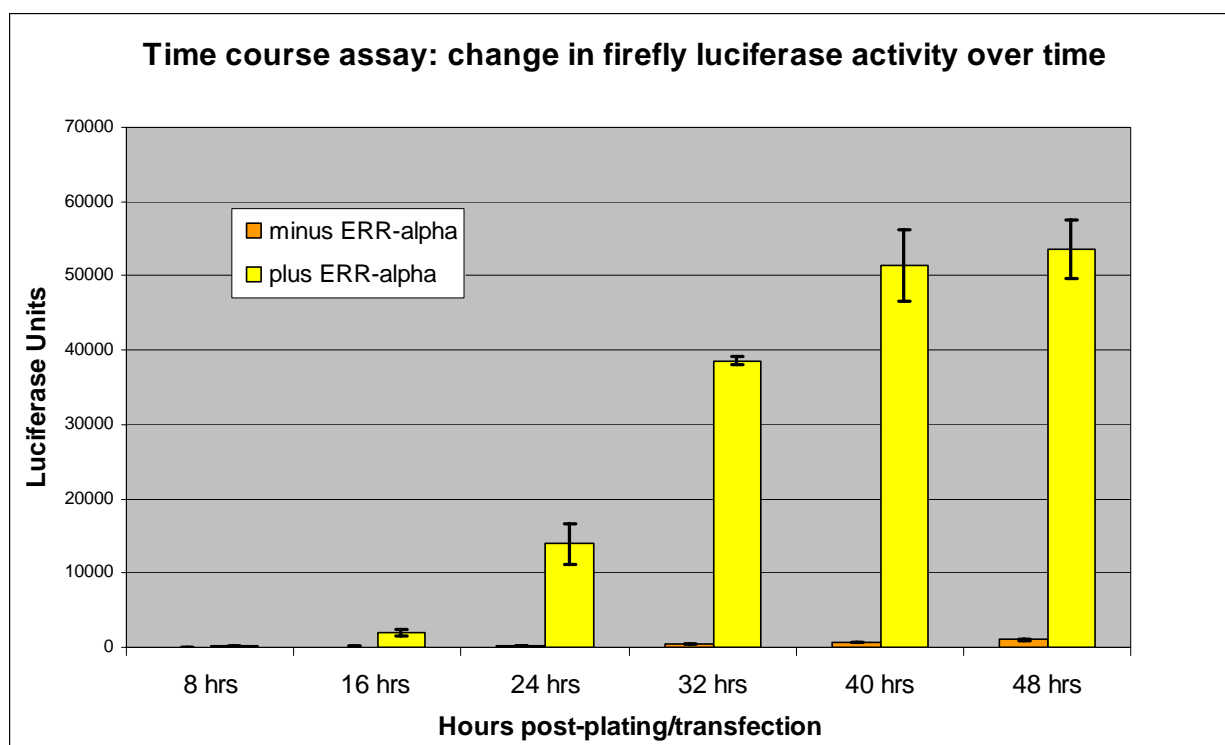
Figure 2. Affect of known compounds on ERRα—GRIP1-dependent luciferase activity.
MCF-7/WS8 cells were transfected in bulk using TransIT-LT1 reagent (Mirus, Madison, WI) and with
the following DNA vectors: p(5x)ERE-ffLuc; pcDNA3.1 (the backbone vector, "-ERR") or truncated
ERRα ("+ERR"); and GRIP1. Twenty-four hours later, transfected cells were treated with known
compounds actinomycin D, alpha-amanitin, cycloheximide, MG-132, resveratrol, or XCT-790 at
various concentrations as indicated. Twenty-four hours after compound addition, treated cells were
harvested. Media was removed from the wells; Bright-Glo luciferase substrate (Promega, Madison,
WI) was added to each well and allowed to incubate for two minutes. Luminescence was read at 700
nm for 1 second per well. Assays were done in quadruplicate. Error bars indicate standard deviation
from the mean.